An Overview of FcγRIIb Mediated HIV and Small Immune Complex Clearance Function of Liver Sinusoidal Endothelial Cells: A Pathway for New Immunotherapies

Undergraduate Research Thesis

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By

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Abstract

The effect of Fc receptors on the regulation of the immune system is well known. In recent years, the so called “inhibitory receptor” FcγRIIb has been shown to be responsible for the clearance mechanism of the liver endothelium, such as clearance of viruses, bacteria, and small immune complexes (SIC). To model the mechanism of clearance via FcγRIIb, antibody-opsonized HIV-like particles (Ab-HIV) were used. It was found that Ab-VLP were cleared considerably faster from circulation than VLP by FcγRIIb on LSEC and the effector mechanism of LSEC FcγRIIb was identified to be endocytosis. From these results, pathological small immune complexes which lead to autoimmune disorders were then studied. A standard treatment for many autoimmune disorders such as Systemic Lupus Erythematosus (SLE), glomerulonephritis, and Idiopathic Thrombocytopenic Purpura (ITP) is intravenous immunoglobulin G (IVIG), however its mechanism is largely unknown. Due to its efficacy and the role FcγRIIb in clearance of SIC, it was hypothesized that the mechanism of IVIG is through the function of the FcγRIIb receptor in order to further understand it in order to further its efficacy for future immunotherapies to treat autoimmune disorders. The effect of IVIG on isolated LSEC from humanized 2B-KIX mice showed an upregulation in the expression of FcγRIIb on the cells surface. Thus, it is clear that the mechanism of action of IVIG on the clearance of SIC from circulation is through the upregulation of FcγRIIb. Modulation of this receptor could lead to new immunotherapies that can more effectively clear HIV and SICs and be a treatment for many other autoimmune disorders.

Dedication

This thesis is dedicated to my immune system, which without, I would have never discovered the world of immunology and the intricacies within it. Thank you for being erratic and leading me to my passion for research and answering the hard questions in life.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisers Dr. Latha Ganesan and Dr. Mark Ruegsegger for the continuous support of my undergraduate research, as well as for their patience, motivation, enthusiasm, and immense knowledge. Thank you for believing in me.

In addition, I would like to thank my fellow labmates, who taught me the skills necessary to complete this thesis, for their unhindering support and friendship: Ozan Suer, Zhili Yao, Charu Tiwari, and Ladan Navari.

Last but not the least, I would like to thank my family: my parents, Thomas Varughese and Nancy Thomas, and sister, Thapasya Thomas, for always supporting my dreams, regardless of some small setbacks, and my partner Isaac Kreitzer for believing in me, listening to me ramble about the immune system, and taking care of me when I needed it most.

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Introduction

Literature Review

FcγRs are integral cell membrane glycoproteins with receptor type-specific distinguishing features. They are the cellular receptors for immunoglobulins and, in humans, consists of four major types, FcγRI(CD64), FcγRII(CD32), FcγRIII(CD16) and FcγRI.[1] Of these, the Fc receptors can be either activation or inhibitory receptors, which transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitory motifs (ITIM), respectively.[2] Of the inhibitory Fc receptors, FcγRIIb has been characterized as one of the key players in the balance of the immune system, as without negative regulators such as the inhibitory Fc receptors, the imbalanced immune response leads to autoimmunity and autoimmune disease.[3]

Previous research in our lab has shown two remarkable, novel findings: first, that the receptor FcγRIIb is found most abundantly in the liver (Fig. 1) and that 90% of the liver's FcγRIIb is found on the surface of the liver sinusoidal endothelial cells (LSEC).[4] These cells are responsible for the clearance function of the liver, including clearance of bacteria, viruses, and small immune

[1]Nimmerjahn, Falk, and Jeffrey V. Ravetch. (2006). Fcγ Receptors: Old Friends and New Family Members. Immunity, Cell Press. www.sciencedirect.com/science/article/pii/S1074761305003833?via%3Dihub.

[2] Ravetch, Jeffrey V., and Lewis L. Lanier. (2000). Immune Inhibitory Receptors. Science, American Association for the Advancement of Science.

[3] Nimmerjahn, Falk, and Jeffrey V. Ravetch. (2006). Fcγ Receptors: Old Friends and New Family Members. Immunity, Cell Press.

[4] Ganesan, L. P., Kim, J., Wu, Y., Mohanty, S., Phillips, G. S., Birmingham, D. J., … Anderson, C. L. (2012). FcγRIIb on liver sinusoidal endothelium clears small immune complexes. Journal of immunology (Baltimore, Md. : 1950), 189(10), 4981–4988. doi:10.4049/jimmunol.1202017

complexes (SIC). The LSECs do this via endocytosis, which they seem to be well equipped for due to their expression of endocytic receptors including scavenger receptors.[5] [6] [7]

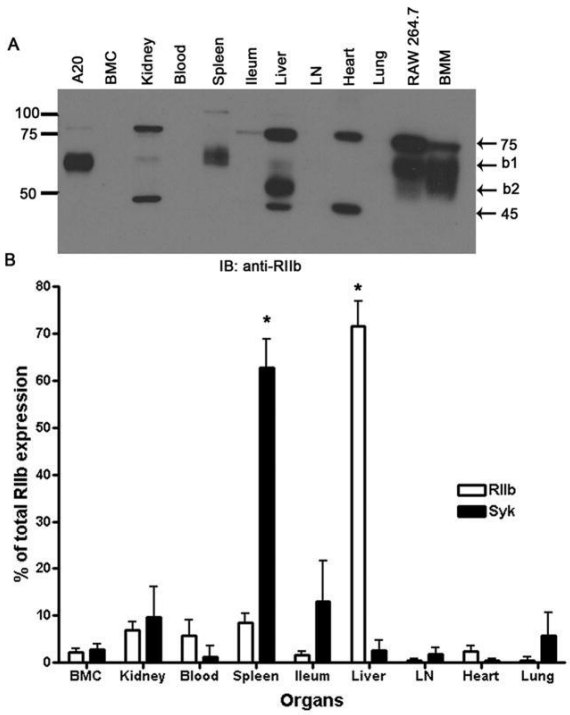


Figure 1: A. An ECL-developed immunoblot using rabbit anti-mouse RIIb antibody showing RIIb expression in molecular weight (kDa) in several tissue and cell lysates. B. Bar graph expressing the means and standard deviations of immunoblot-derived band densities, after factoring total organ weight for both RIIb isoforms and Syk from all organs (n=3 WT mice). The asterisks indicate that the expression levels of Syk and RIIb in spleen and liver, respectively, are statistically significantly different from the average of all other organs ($P<0.001$). (Ganesan, et al. 2012).

[5] Yao, Z., Mates, J. M., Cheplowitz, A. M., Hammer, L. P., Maiseyeu, A., Phillips, G. S., … Ganesan, L. P. (2016). Blood-Borne Lipopolysaccharide Is Rapidly Eliminated by Liver Sinusoidal Endothelial Cells via High-Density Lipoprotein. Journal of immunology (Baltimore, Md. : 1950), 197(6), 2390–2399. doi:10.4049/jimmunol.1600702

[6] Elvevold, K. , Simon-Santamaria, J. , Hasvold, H. , McCourt, P. , Smedsrød, B. and Sørensen, K. K. (2008), Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity. Hepatology, 48: 2007-2015. doi:10.1002/hep.22527

[7] Sørensen, K. K., Simon-Santamaria, J. , McCuskey, R. S. and Smedsrød, B. (2015). Liver Sinusoidal Endothelial Cells. In Comprehensive Physiology, R. Terjung (Ed.). doi:10.1002/cphy.c140078

Antibody opsonized HIV (ab-HIV) falls into the category of small immune complexes (SIC) as these complexes are the size appropriate for receptor-mediated endocytosis by LSEC.[8] Our lab was focused on how neutralizing antibodies (NAbs) suppress HIV infection by accelerating viral clearance from blood circulation, in addition to neutralization. NAbs bind and inactivate viruses through their Fab regions. Thus, NAbs block HIV attachment to target cells by interfering with the interaction between HIV and its cellular receptors, such as the CD4.[9]

Research Significance

In recent years, new insight on how the body eliminates SIC from the blood has come to light. It was long known that pathogenic SICs, which deposit in organs like the kidneys, cause inflammation and diseases, such as Systemic Lupus Erythematosus (SLE), glomerulonephritis and many other autoimmune disorders. Normally, the majority of SIC are eliminated harmlessly by the liver before they can crosslink and form large immune complex, which eventually deposit in kidneys, resulting in the clinical manifestations of SLE. In addition, intravenous immunoglobulinG (IVIG), is a common treatment for SLE as well as other autoimmune diseases such as idiopathic thrombocytopenic purpura. However, the mechanism of IVIG for SIC removal is largely unknown. Given that FcγRIIb is known to clear SICs, I hypothesized that IVIG upregulates the expression of FcγRIIb on the surface of LSECs.

This project has broad relevance to public health in that it will define the novel mechanism mediated by FcγRIIb in liver endothelium to eliminate rapidly and efficiently the circulating

[8] Mayr, L. M., Su, B., & Moog, C. (2017). Non-Neutralizing Antibodies Directed against HIV and Their Functions. Frontiers in immunology, 8, 1590. doi:10.3389/fimmu.2017.01590

[9] Burton, D. Antibodies, viruses and vaccines. (2002). Nat Rev Immunol **2,** 706–713 doi:10.1038/nri891

immune complexes that cause painful and debilitating autoimmune diseases. The outcome of these studies will improve our understanding of the mechanism of action of IVIG in vivo and present novel methods to increase the efficacy of IVIG or even innovate new antibody-based immunotherapy that works more effectively.

Methodology

To evaluate the clearance of antibody opsonized HIV (Abs-HIV) and understand the mechanism of IVIG, we obtained an engineered mouse strain expressing human rather than mouse FcγRIIb (2B-KIX: FcγRIIb-Knock-In; Xencor).

To identify the organ responsible for the enhanced clearance of Ab-HIV, we quantified the organ distribution of intravenously infused mixtures of HIV immunoglobulin (HIVIG) and Alexa 594 conjugated-virus-like-particle (594-VLP) following previously published protocols[10] [11]. The liver, heart, lung, spleen, and kidney were harvested 10 minutes after infusion, after euthanizing the mice. Approximately 100 mg of each organ was lysed in tissue cell lysis buffer and fluorescence intensity was determined using an EnspireTM multimode plate reader (Perkin Elmer). The percentage distribution of HIVIG-594 was calculated by factoring the total weight of each organ.

[10] Ganesan, L. P., Mohanty, S., Kim, J., Clark, K. R., Robinson, J. M., & Anderson, C. L. (2011). Rapid and efficient clearance of blood-borne virus by liver sinusoidal endothelium. *PLoS pathogens*, *7*(9), e1002281. doi:10.1371/journal.ppat.1002281

[11] Ganesan, L. P., Kim, J., Wu, Y., Mohanty, S., Phillips, G. S., Birmingham, D. J., … Anderson, C. L. (2012). FcγRIIb on liver sinusoidal endothelium clears small immune complexes. Journal of immunology (Baltimore, Md. : 1950), 189(10), 4981–4988. doi:10.4049/jimmunol.1202017

To determine the efficacy of the FcγRIIb in the clearance of HIV, male, 12-week-old, 2B-KIX and FcγRIIb KO mice (n = 4) were both infused by tail vein with Ab-HIV prepared using 5x1010 HIV-VLP and 50mg/kg of HIVIG. The HIV particles still in circulation were quantified at 30 seconds, 2, 3, 5, and 10 minutes using heparinized capillary tubes by taking blood samples via retro-orbital plexus. Blood obtained at each time point was diluted and the HIV p24 concentration was determined as pg/mL using p24 ELISA (Zeptometrix).

To determine the effects of IVIG on the function of the LSECs, the expression of FcγRIIb was quantified using flow cytometry. Fresh LSECs were prepared from 2B-KIX mice (n = 3) and treated with IVIG and BSA as a control. The concentration of IVIG and the in vitro treatment was defined by literature.[12] [13] [14]

Table 1.

	1	2	3
A	Resting Cells Primary + Secondary	Resting Cells Isotype + Secondary	Resting Cells Cells Alone
B	Cells Treated w/ IVIG Primary + Secondary		
C	Cells Treated w/ BSA Primary + Secondary		

Table 1 shows the plating technique for overnight treatment. Resting cells were suspended in DMEM F12 while IVIG treated cell and BSA cells were treated with their respective supplements at the same concentration in DMEM F12. The cells were incubated at 5% CO and 37 degrees C for 8 hours.

[12] Siragam, V., Crow, A. R., Brinc, D., …. Lazarus, A. H. (2006). Intravenous immunoglobulin ameliorates ITP via activating Fcg receptors on dendritic cells"

[13] Nagelkerke, S. Q., Dekkers, G., Kustiawan, I., … Kuijpers, T. W. (2014). Inhibition of FcγR-mediated phagocytosis by IVIg is independent of IgG-Fc sialylation and FcγRIIb in human macrophages. Blood, 124(25), 3709-3718.

[14] Nagelkerke, S. Q., & Kuijpers, T. W. (2015). Immunomodulation by IVIg and the Role of Fc-Gamma Receptors: Classic Mechanisms of Action after all?. Frontiers in immunology, 5, 674. doi:10.3389/fimmu.2014.00674

Animals: Transgenic mice expressing human FcγRIIb, 2B-KIX, from Xencor were bred and maintained. FcγRIIb knock-out mice in C57BL/6 background (Dr. Jeffrey Ravetch) were purchased from The Jackson Laboratory. All studies were approved by The Ohio State University Institutional Animal Care and Use Committee and all procedures were in accordance with their guidelines. All surgery was performed under Isoflurane anesthesia, and all efforts were made to minimize suffering.

Results

To obtain purified LSEC preparations, two negative selection steps were taken, using F4/80 microbeads and anti-CD45 microbeads, to remove Kupffer cells (KC) and other leukocytes, respectively, from liver cell suspensions. Then, a positive selection for CD146 cells yielded in a pure LSEC preparation (Fig 2).

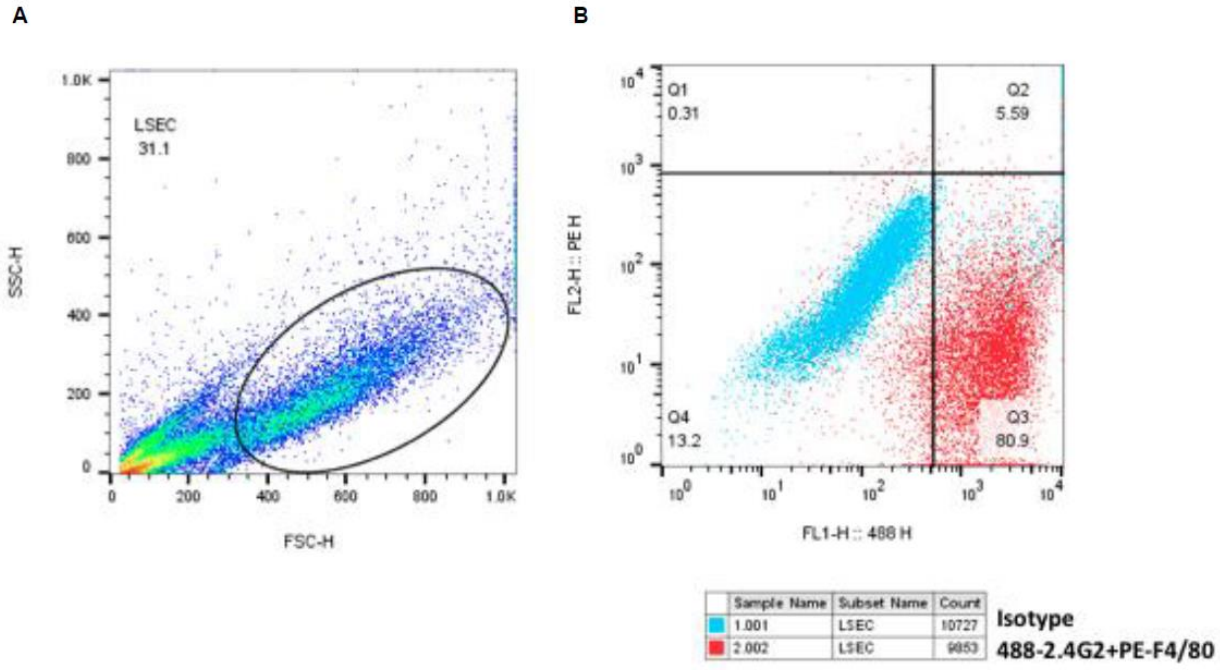


Figure 2. Flow cytometry dot-plots show that the purity of the isolated cells, as evidenced in panel A. Panel B compares the isotype with shows that the isolated cells express 2.4G2 and are F/80-, which have been previously determined to be LSEC markers.

In order to test the phenotype of purified LSEC from humanized mice, the specificity of anti-human FcγRIIb mAb 4F5[15] labeled with fluor Alexa 594 was tested for specificity for human FcγRIIb using mouse B cell line (A20), a human B cell line (Raji), and a cell line that lacks FcγR (HEK) (Fig 3).

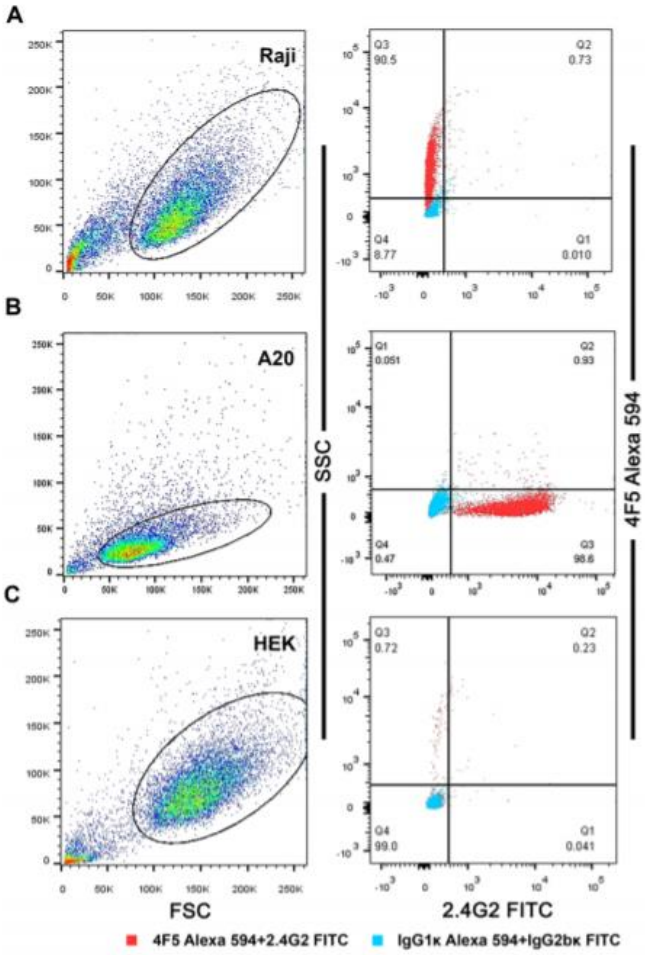


Figure 3: A. Representative flow cytometric acquisition plot showing gated cell lines; namely, Raji (Panel A), A20 (Panel B), and HEK (Panel C) in the first column; and two-color flow cytometric analysis of gated population for 2.4G2 FITC plus 4F5 Alexa 594 in the second column. The results are representative of 4 different experiments and mice. In A-C, events from isotype controls are represented as blue dots and from primary antibodies as red dots. The average percentage of events showing single or double positive expression from three different experiments is indicated in respective quadrants.

[15] Su, W. T., Rutigliano, D. N., Gholizadeh, M. , Jarnagin, W. R., Blumgart, L. H. and La Quaglia, M. P. (2007), Hepatic metastasectomy in children. Cancer, 109: 2089-2092. doi:10.1002/cncr.22650

The presence of fenestrae is considered the gold standard morphologic feature distinguishing LSEC from other cells of the liver. LSEC purified by the triple immunomagnetic separation shown the typical fenestrae in the membrane by SEM (Fig 4).

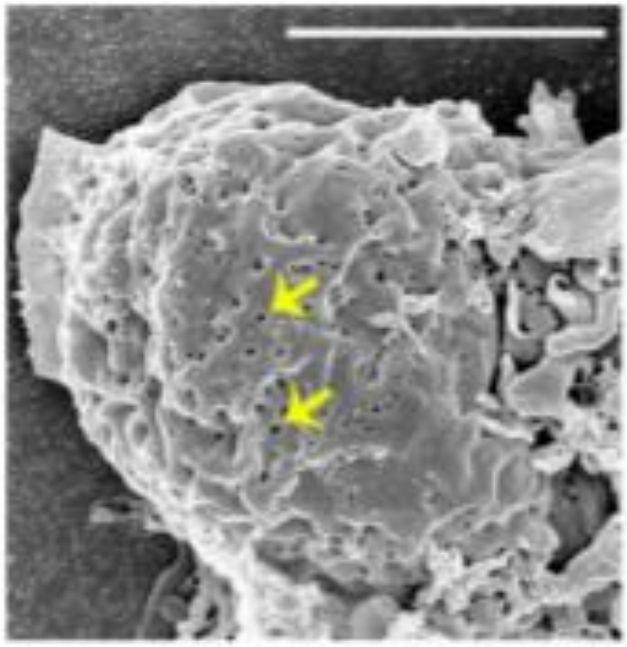


Figure 4. The picture shows the SEM image of LSEC from a 2B-KIX mouse. Arrows point fenestrae. Note folded and layered cell membranes of LSEC. The scale bar indicates 5μm.

Ten minutes after infusions, HIV particles were recovered 86±9% in liver, 2.2±1.1% in lung, 0.1±0.3% in spleen, 1.8±1.3% in kidney and 9.0±9% in blood circulation suggesting strongly that liver is the major organ responsible for clearing circulating Ab-HIV (Fig 5).

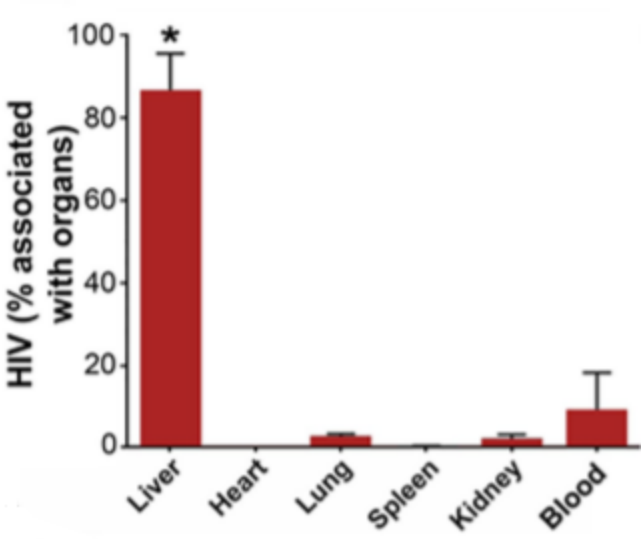


Figure 5: The bar graph shows the distribution percentage of Ab-HIV to various organs including blood after factoring total organ weight ± SD from 3 different mice. The * represents a p value of 0.05 by students t-test.

Compared to the FcRIIb KO mice, the 2B-KIX (humanized FcγRIIb) mice had less HIV particles in circulation at every time point over a period of 10 minutes, thus indicating that the FcγRIIb receptor is mediating the clearance of the HIV particles.

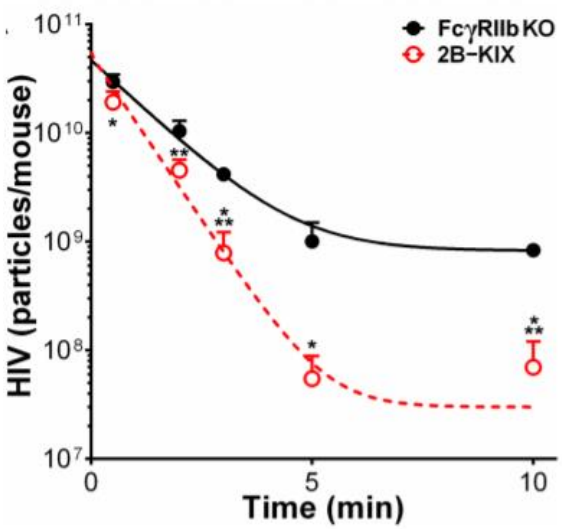
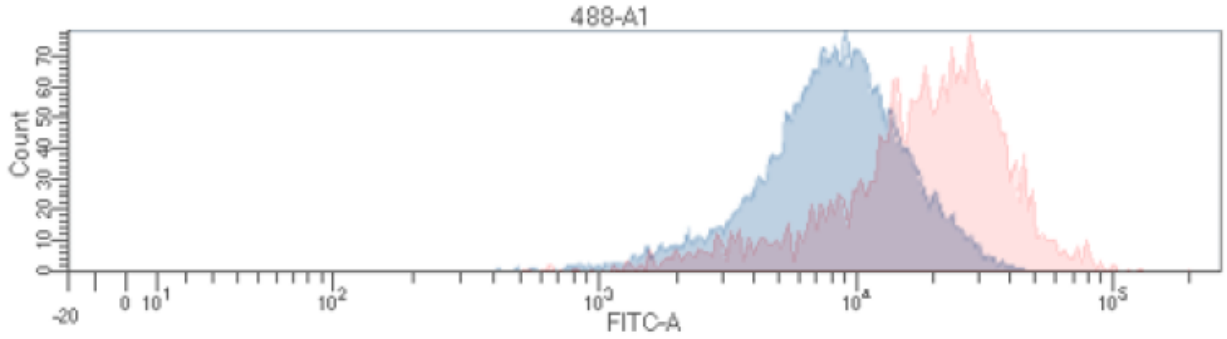


Figure 6. Clearance kinetics showing disappearance of Ab-HIV from blood circulation of 2B-KIX mouse (red open circle) and FcγRIIb KO (black closed circle). The curve describes the HIV-VLP particle numbers in blood per mouse cleared over ten min time ± SD using data averaged from 4 different mice. Values of all significant correlations ($p<0.05$) are given with degree of significance indicated (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) by student's t-test.

With this knowledge of Ab-HIV being cleared via mechanism of the FcγRIIb receptor, it became clearer that IVIG must be clearing SIC via the same mechanism. After treatment of the cells with IVIG and respective controls for 8 hours, a clear upregulation of FcγRIIb was seen (Fig 7).

A



B

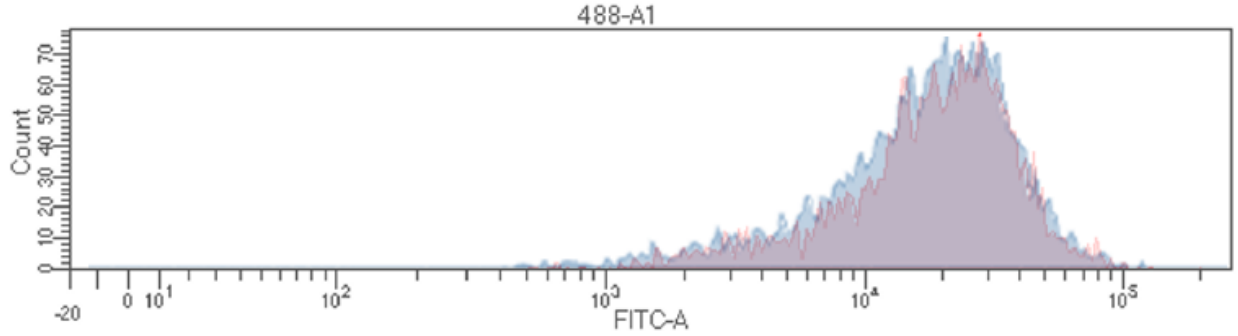


Figure 7. A: Expression of FcγRIIb on cells treated by IVIG overnight (blue) overlaid on control cells (n = 3). B: Expression of FcγRIIb on cells treated with BSA (control) overnight (blue) overlaid on control cells (n = 3).

Conclusion

It is clear that FcγRIIb is the leading mediator or clearance of viruses and SIC. This is seen through the efficacy of clearance of Ab-HIV. This conclusion can lead to multiple studies of clearance mediated by FcγRIIb, including that of pathological SIC which may cause a host of autoimmune disorders. The result of IVIG, a common drug used to treat a multitude of various

autoimmune disorders, used to treat LSEC led to an upregulation of FcγRIIb, implying that its mechanism of action is also through this receptor. Modulation of this receptor could lead to new immunotherapies that can more effectively clear HIV and SICs and be a treatment for many other autoimmune disorders.

Contributions

I have standardized the protocol to isolate pure LSECs from mice needed for the aforementioned experiments. Prior, there was no good way to purify LSEC from the liver of mice without contamination from other cells and loss of the majority of the LSEC. The method that I have standardized uses a triple immuno-magnetic cell separation, which uses microscopic magnets, called microbeads, that bind to unique receptors on the cells of the liver in order to separate them based on positive and negative selection, which ultimately produces a pure and enriched LSEC sample from each mouse liver. This current method yields, at most, 5 million LSECs per mouse, whereas previous methods would yield a mere 0.5- 1 million LSECs per mouse. A fully detailed protocol is attached in the Appendix Section.

Additionally, I prepared the HIV-VLP via transfection of HEK cells for all experiments regarding the clearance mechanism of Ab-HIV. I also completed the overnight studies using IVIG independently.

Future work

Many future experiments need to be done to fully understand the mechanism of action of IVIG mediated clearance. First, the experiment needs to be repeated in order to collect the cells and evaluated them using RT-PCR. This will show, undeniably, the increase of gene expression for FcγRIIb. In addition, future experiments should include repeating with multiple concentrations

of IVIG to understand dosing requirements, comparison the SIC uptake by LSECs treated with and without IVIG, *in vivo* kinetic studies using IVIG treatment of SIC on WT mice and RIIb KO mice and repetition of all *in vitro* experiments with a human cell line that expresses the correct FcγRIIb phenotype.

Appendices

Appendix A: Liver Dissociation Protocol to Obtain LSEC via Immunomagnetic Separation

Note: Operate MACSmix Tube Rotator on permanent run at a speed of approximately 12 rpm.

A. Liver dissociation protocol

1. Prepare dissociation mix by pipetting 4.7 mL GentleMACS DMEM into a gentleMACS C Tube.
    i. Use ~200 uL of this GentleMACS DMEM to wash the inside of the following enzyme vials in order to ensure maximum enzyme volume.
2. Add 200 µL Enzyme D solution, 100 µL Enzyme R solution, and 20 µL Enzyme A solution to the C tube. → dissociation mix **(see appendix for aliquoting)**
3. Incubate dissociation mix for 30 minutes at 37 °C in an incubator or 15 minutes in a water bath.
    i. Get the mouse during this incubation and excise liver.
4. Dissect liver from mouse
    i. Remove gallbladder with forceps before dissecting the mouse liver and resect connective tissue.
5. Rinse liver with GentleMACS DMEM by placing in petri dish.
6. Transfer liver into the C Tube containing the dissociation mix.
7. Tightly close C Tube and attach it upside down onto the sleeve of the GentleMACS Dissociator.
    i. Ensure that the sample material is located in the area of the rotor/stator.
    ii. Close C Tube tightly beyond the first resistance.
8. Run the GentleMACS Program m_liver_03.
    i. Repeat no more than 3 times
    ii. Ensure dissociation of all lobes
9. After termination of the program, detach C Tube from the GentleMACS Dissociator.
10. Incubate sample for 30 minutes at 37 °C under continuous rotation using the MACSmix Tube Rotator.
    i. Make the 1 X lysis buffer in this step

11. Attach C Tube upside down onto the sleeve of the GentleMACS Dissociator.
12. Run the GentleMACS Program m_liver_04.
13. After termination of the program, detach C Tube from the GentleMACS Dissociator.
14. Pour the cell suspension to a MACS SmartStrainer (100 µm).
15. Wash the filter with 5 mL GentleMACS DMEM with stable glutamine and P/S and add to the smart strainer.
    i. For maximum cell recovery, rinse the used C Tube with ~5 mL GentleMACS DMEM before transferring to filter.
16. Discard the filter and centrifuge cell suspension at 60 × g for 5 minutes to pellet the hepatocytes.
    i. Collect the supernatant without disturbing the pellet at all.
17. Centrifuge at 2600 RPM for 10 minutes to pellet the NPCs.
    i. Aspirate supernatant.
18. Resuspend cells in 10 mL of 1X Red Blood Cell Lysis Solution
19. Vortex for 5 seconds and incubate for 2 minutes at room temperature.
20. Centrifuge at 2600 RPM for 10 minutes at room temperature to pellet NPCs.
    i. Aspirate supernatant completely.
21. Resuspend the cell pellet in an appropriate buffer and proceed to further applications.

B. Immunomagnetic Separation Protocol

I. Magnetic Labeling: F4/80 Conjugated Microbeads

1. Resuspend NPCs in 400 ul MACS buffer, keep cells on ice.
2. Add 10 ul of F4/80 Conjugated Microbeads and incubate for 15 min. in 4°C (IN BEER COOLER, NOT ON ICE!)
    a. Wash by directly adding 10ml MACS buffer to primary antibody and mix
    b. Centrifuge cells at 2600rpm for 5 min. at 4°C. Aspirate supernatant
3. To the pellet add 500ul of MACS buffer
    a. If cells are clumping it is ok to resuspend cells in 1ml of MACS buffer

II. Magnetic Separation: F4/80+ and F4/80- Selection

4. Place the LS column in the magnetic field
    a. Prongs/projections pointed towards you

5. Place an appropriately labelled collection tube (50 ml falcon tube) below the column
6. Prepare the LS column (Cat #130-042-401) by rinsing with 3ml MACS buffer
7. Place the 30um Pre-Separation Filter (Cat #120-002-220) on top of the column
    a. Place another appropriately labelled collection tube below column (F4/80- selection). This fraction will contain LSECs, NK cells, dendritic cells, etc.
8. Apply cells to pre-separation filter, allow the cells to enter the column when there is a meniscus in the column
    a. Do not let the column get dry.
    b. Wash the column 3 times with 3ml MACS buffer for a total of 9ml. Add the next 3 ml when there is a slight meniscus on top of the magnetic part of the column
    c. Pipet MACS buffer slowly by touching the pipet to the wall of the pre-separation filter. Be careful not to induce any air bubbles.
9. Cap "F4/80- selection" tube and centrifuge at 2600rpm for 5 min. at 4°C
10. While tube is centrifuging, remove column from magnet and place in another appropriately labelled "F4/80+ selection"15 ml falcon tube and remove pre-separation filter
    a. Tube labelled "**F4/80+ selection**" will contain **KCs**
11. To the "F4/80+ selection" tube, pipet 5ml MACS buffer into the column and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column
12. Centrifuge "F4/80+ selection" tube at 2600rpm for 3 min. at 4°C and resuspend in 500ul MACS buffer and store on ice until you are ready to start the flow protocol
    a. Count cells this should be the KCs

III. Magnetic Labeling: CD45 Microbeads

13. Resuspend NPCs from the F4/80- selection in 200 ul of MACS buffer per 10^7 cells
14. Add 10 ul of CD45 Microbeads (Cat #130-052-301) per 10^7 cells to the cell suspension above and incubate for 15 min. in 4°C **(IN BEER COOLER, NOT ON ICE!)**
15. Wash cells + microbeads by adding 6ml MACS buffer
    a. Centrifuge at 2600 rpm for 5 min at 4°C
    b. Aspirate off supernatant
16. Resuspend cells in 500ul of MACS buffer
    a. If cells are clumping it is ok to resuspend cells in 1ml of MACS buffer

IV. Magnetic Separation: CD45- Selection

17. Place the LS column in the magnetic field
    a. Prongs/projections pointed towards you
18. Place an appropriately labelled collection tube (50 ml falcon tube) below the column
19. Prepare the LS column by rinsing with 3ml MACS buffer
20. Place the 30um Pre-Separation Filter on top of the column
21. Place another tube labelled "CD45- and F4/80-" selection below column (eluent)
22. Apply cells to pre-separation filter
23. Wash the column 3 times with 3ml MACS buffer for a total of 9ml
24. Discard the column as we do not need CD45 + cells.
25. Cap "CD45- and F4/80-" tube and centrifuge at 2600rpm for 3 min. at 4°C

V. ***Magnetic Labeling: CD146 (LSEC) Microbeads***

26. Resuspend "F4/80- & CD45-" cells in 200 ul of MACS buffer per 10^7 cells
27. Add 20 ul of CD146 (LSEC) Microbeads (Cat # 130-092-007 Miltenyi biotech) to the cell suspension above and incubate for 15 min. in 4°C **(IN BEER COOLER, NOT ON ICE!)**
28. Wash cells + microbeads with 6ml MACS buffer
    a. Centrifuge at 2600 rpm for 3 min (if need longer can spin for 10 min) at 4°C
    b. Aspirate off supernatant
29. Resuspend cells in 500ul of MACS buffer

VI. ***Magnetic Separation: (1) F4/80-, CD45- and CD146- Selection (2) F4/80-, CD45- and CD146+ Selection***

30. Place the LS column in the magnetic field
    a. Prongs/projections pointed towards you
31. Place an appropriately labelled collection tube (50 ml falcon tube) below the column
32. Prepare the LS column by rinsing with 3ml MACS buffer
33. Place the 30um Pre-Separation Filter on top of the column
34. Place another tube labelled "F4/80- and CD45- and CD146-" 50 mL falcon tube below column
35. Apply cells to pre-separation filter
36. Wash the column 3 times with 3ml MACS buffer for a total of 9ml

37. Discard the tube labelled "F4/80- and CD45- and CD146-" selection
38. Remove column from magnet and place in another appropriately labelled "**F4/80- and CD45- and CD146+ selection"**
    a. **<u>These are LSECs (*F4/80- and CD45- and CD146+ selection)*</u>**
39. To the column add 5ml MACS buffer into the column and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column
40. Centrifuge "F4/80- and CD45- and CD146+ selection" tube at 2600rpm for 3 min. at 4°C and resuspend in 500ul MACS buffer and store on ice. Count cells this should **<u>be the LSECs</u>**
41. **<u>If you are doing IF</u>**
    a. Pellet the cells at 2600rpm for 3 min and resuspend in 1 ml 4%PFA in PBS FACS (from 20%)/mice cells
    b. Incubate for 15 min ROOM TEMPRATURE
    c. Pellet the cells at 2500rpm for 3 min and resuspend in 1 ml PBS FACS
    d. Repeat step c
    e. Resuspend in PBS FACS 1 ml. Cover with aluminum foil. Store in 4C

Appendix B: Reagent Preparation for Liver Dissociation Protocol

Appendix: Reagent preparation

1. Prepare Enzyme D by reconstitution of the lyophilized powder in the vial with 3 mL DMEM with stable glutamine and P/S at room temperature.
    i. Do not try to resuspend by pipetting or vortexing.
    ii. Invert vial after closing and wait 5–10 minutes to dissolve the pellet.
    iii. Enzyme D should be sterile filtered with a small syringe filter prior to aliquoting.
    iv. Prepare aliquots of 200 uL to avoid repeated freeze-thaw-cycles. Store aliquots at –20 °C.
    v. This solution is stable for 6 months after reconstitution.
2. Prepare Enzyme R by reconstitution of the lyophilized powder in the vial with 2.7 mL of DMEM with stable glutamine and P/S at room temperature.

i. Prepare aliquots of 100 uL to avoid repeated freeze-thaw-cycles. Store aliquots at –20 °C.
ii. This solution is stable for 6 months after reconstitution.
iii. Make sure to thoroughly mix this suspension immediately before withdrawing the required reaction volume!

3. Prepare Enzyme A by reconstitution of the lyophilized powder in the vial with 1 mL DMEM with stable glutamine and P/S at room temperature.
    i. Do not vortex.
    ii. Prepare aliquots of 20 uL to avoid repeated freeze-thaw-cycles. Store aliquots at –20 °C.
    iii. This solution is stable for 6 months after reconstitution.
4. Preparation of 1× Red Blood Cell Lysis Solution
    i. Dilute Red Blood Cell Lysis Solution (10×) 1:10 with double distilled water (ddH_2O), for example, dilute 1 mL of Red Blood Cell Lysis Solution (10×) with 9 mL of ddH_2O.
    ii. Do NOT dilute with deionized water.
    iii. Store the prepared 1× Red Blood Cell Lysis Solution at room temperature.
    iv. Discard unused solution at the end of the day.
5. Preparation of GentleMACS DMEM
    i. Start with incomplete DMEM
    ii. Add 1% P/S and 1% L-glut

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Figures

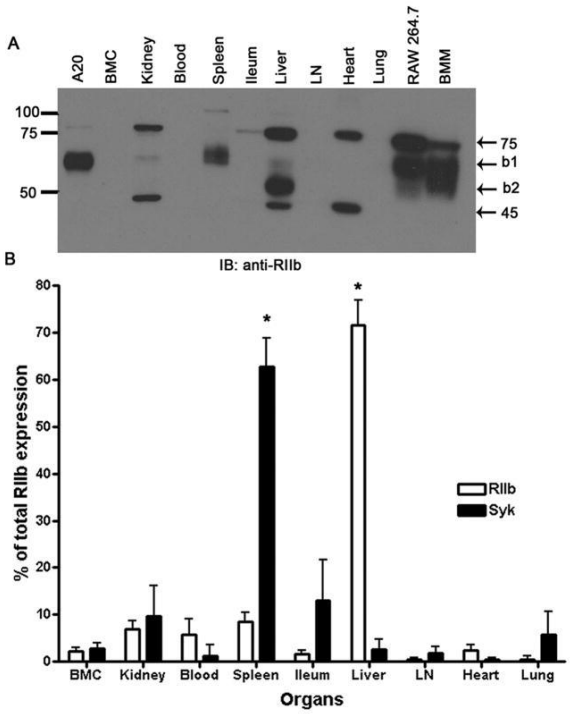

Figure 1: A. An ECL-developed immunoblot using rabbit anti-mouse RIIb antibody showing RIIb expression in molecular weight (kDa) in several tissue and cell lysates. B. Bar graph expressing the means and standard deviations of immunoblot-derived band densities, after factoring total organ weight for both RIIb isoforms and Syk from all organs (n=3 WT mice). The asterisks indicate that the expression levels of Syk and RIIb in spleen and liver, respectively, are statistically significantly different from the average of all other organs (P<0.001). (Ganesan, et al. 2012).

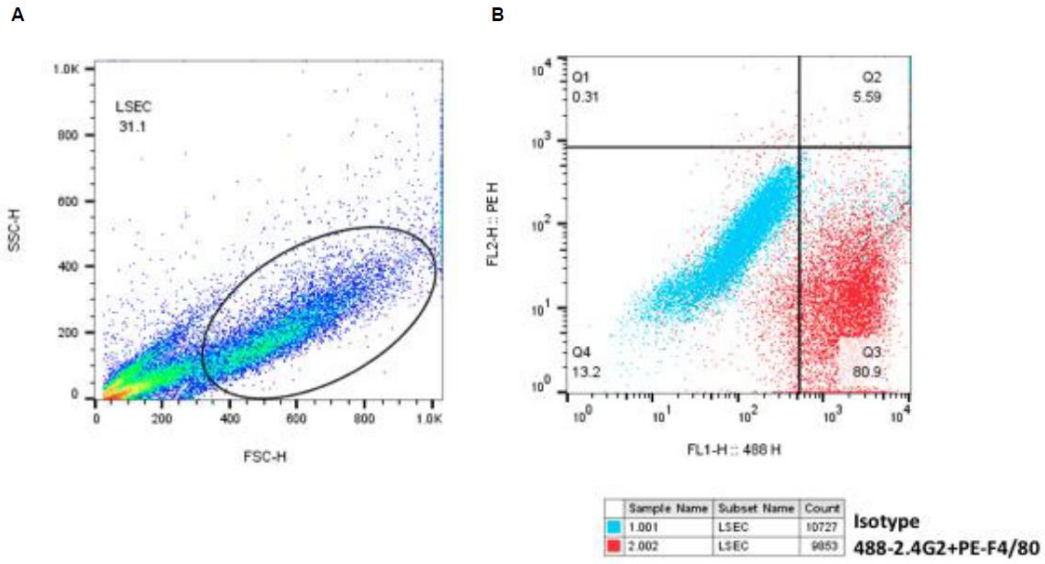

Figure 2. Flow cytometry dot-plots show that the purity of the isolated cells, as evidenced in panel A. Panel B compares the isotype with shows that the isolated cells express 2.4G2 and are F/80-, which have been previously determined to be LSEC markers.

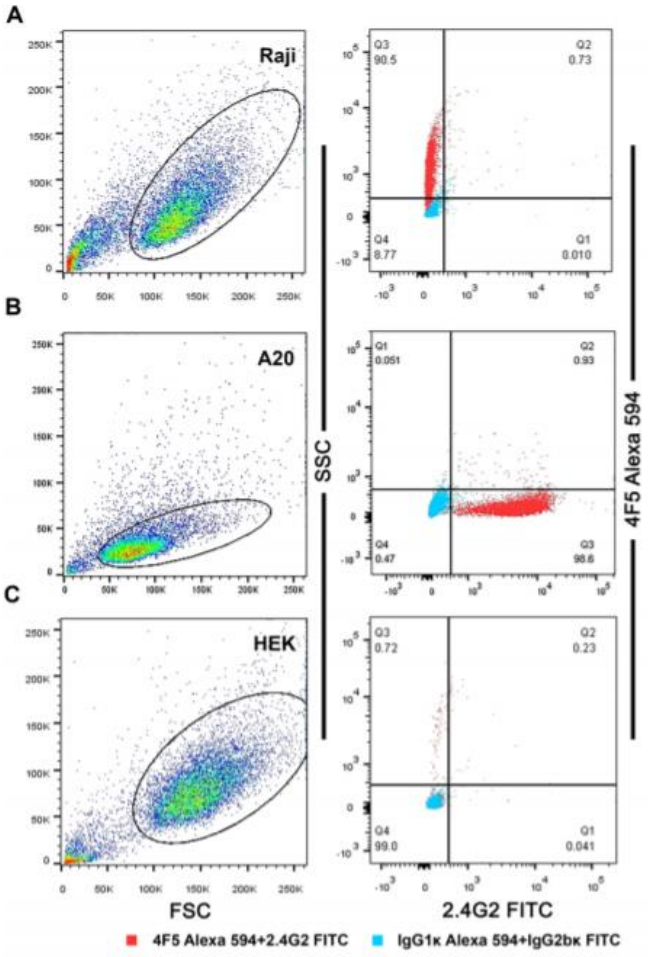

Figure 3: A. Representative flow cytometric acquisition plot showing gated cell lines; namely, Raji (Panel A), A20 (Panel B), and HEK (Panel C) in the first column; and two-color flow cytometric analysis of gated population for 2.4G2 FITC plus 4F5 Alexa 594 in the second column. The results are representative of 4 different experiments and mice. In A-C, events from isotype controls are represented as blue dots and from primary antibodies as red dots. The average percentage of events showing single or double positive expression from three different experiments is indicated in respective quadrants.

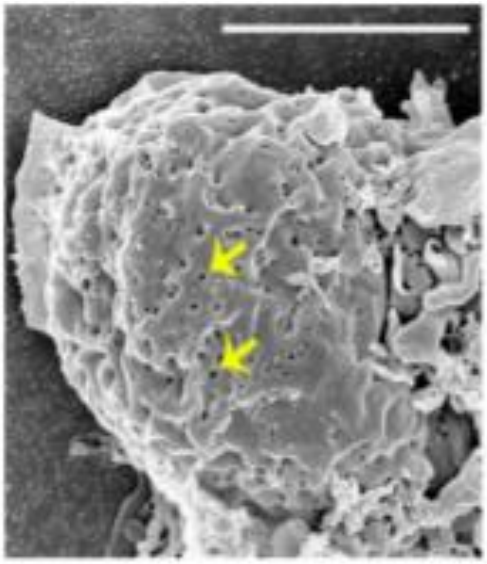

Figure 4. The picture shows the SEM image of LSEC from a 2B-KIX mouse. Arrows point fenestrae. Note folded and layered cell membranes of LSEC. The scale bar indicates 5µm.

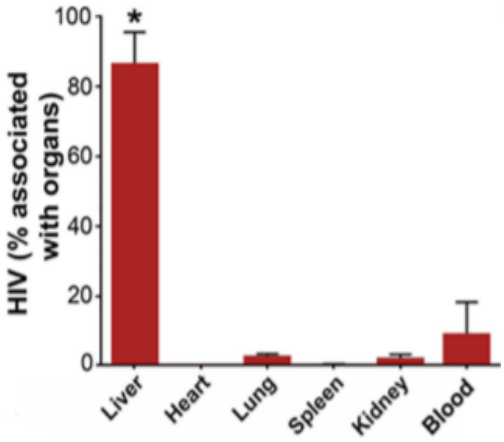

Figure 5: The bar graph shows the distribution percentage of Ab-HIV to various organs including blood after factoring total organ weight ± SD from 3 different mice. The * represents a p value of 0.05 by students t-test.

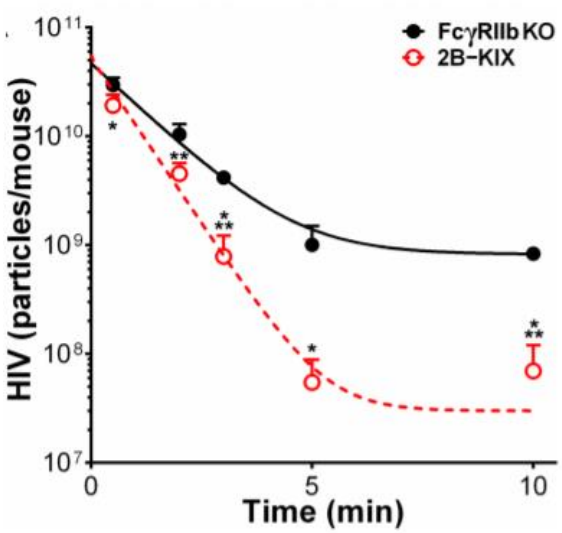

Figure 6. Clearance kinetics showing disappearance of Ab-HIV from blood circulation of 2B-KIX mouse (red open circle) and FcγRIIb KO (black closed circle). The curve describes the HIV-VLP particle numbers in blood per mouse cleared over ten min time ± SD using data averaged from 4 different mice. Values of all significant correlations ($p<0.05$) are given with degree of significance indicated (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) by student's t-test.

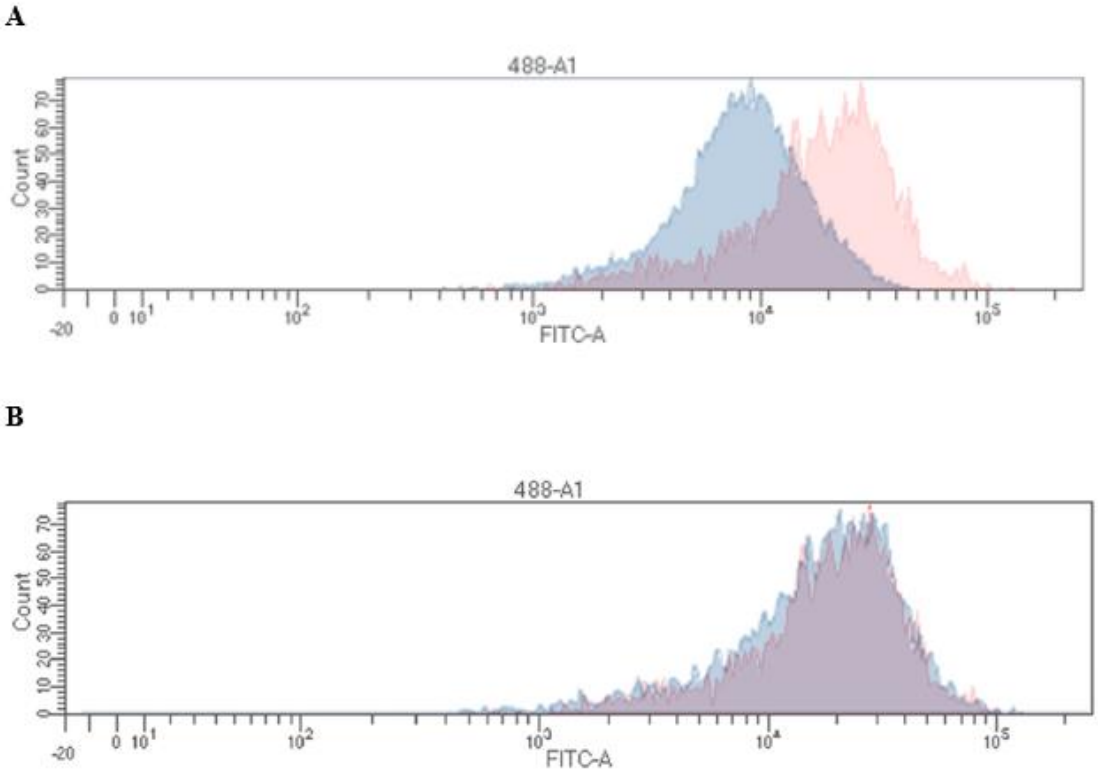

Figure 7. A: Expression of FcγRIIb on cells treated by IVIG overnight (blue) overlaid on control cells (n = 3). B: Expression of FcγRIIb on cells treated with BSA (control) overnight (blue) overlaid on control cells (n = 3).